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Note

Separation of glyoxylated hemoglobin with varying oxygen affinity by polyanion fast protein liquid chromatography

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Considerable research effort has recently been directed toward the modification of stroma-free hemoglobin (SFH) to allow its use as a basis for blood substitutes[1]. One of the properties which requires modification is its oxygen-binding affinity, which is excessive in the absence of certain ligands within the red cell. For this reason, the polyphosphate binding site of hemoglobin has recently been modified with glyoxylate[2]. The oxygen affinity of glyoxylated stroma-free hemoglobin (G-SFH) is similar to that of pyridoxal phosphate-modified hemoglobin, *i.e.* its P_{50}, the partial pressure of oxygen required to saturate 50% of the hemoglobin, is approximately twice that of unmodified hemoglobin. Hedlund *et al.*[3] have suggested that glyoxylate modification is preferable to pyridoxal phosphate modification. These authors point out that aside from reagent cost considerations, glyoxylation does not require that hemoglobin be in the deoxy state, whereas pyridoxal phosphate modification does.

There is heterogeneity in glyoxylated hemoglobin preparations due to variations in the extent of glyoxylation. Right-shifting of the oxygen dissociation curve is proportional to the extent of glyoxylation. A method for quantitating the extent of glyoxylation is therefore of paramount importance in the successful scaling-up of production of a modified hemoglobin with optimal oxygen binding properties.

In the present communication, a fast protein liquid chromatographic (FPLC) method is described which separates optimally glyoxylated hemoglobin from a mixture of native and partially glyoxylated hemoglobin. The hemoglobin fractions were characterized by their oxygen-binding affinity and their electrophoretic mobility on cellulose acetate.

MATERIALS AND METHODS

SFH and G-SFH, prepared by the method of Acharya *et al.*[2] were obtained from the University of Minnesota. The methemoglobin content was measured with a Corning 2500 CO oximeter and found to be 10.6 and 3.6% in SFH and G-SFH respectively. Samples not exceeding 50 mg hemoglobin in solution were applied to an HR 10/10 column (100 × 10 mm I.D.) packed with 10 ml of polyanion SI-17 μm (Pharmacia) equilibrated with 0.02 *M* Tris–HCl, pH 7.54 (buffer A). The column

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was washed with 2 bed volumes of buffer A at a flow-rate of 1 ml/min. Buffer B (0.5 *M* sodium chloride in buffer A) was introduced via a linear gradient to elute the second fraction. Fractions were pooled, concentrated, dialysed against Ringer's buffer, pH 7.4, and hemoglobin concentration normalized to 50 mg/ml for application to a Hem-O-Scan oxygen dissociation analyzer. Cellulose acetate electrophoresis was conducted for each of the fractions in barbital buffer, pH 8.8 (high-resolution buffer, Gelman).

RESULTS AND DISCUSSION

Fig. 1 shows that G-SFH is separated into two fractions on the polyanion column. We attribute this differential binding to differences in the extent of masking of the N-terminal amino groups of the alpha- and beta-subunits of hemoglobin by glyoxylation. The greater the extent of glyoxylation, therefore, the stronger the binding of the hemoglobin to the column; the net negative charge on optimally modified hemoglobin is consistent with the anion-exchange behaviour of fraction 2. (This peak appears to have two shoulders, possibly indicating subfractions; we have made no attempt to separate these.)

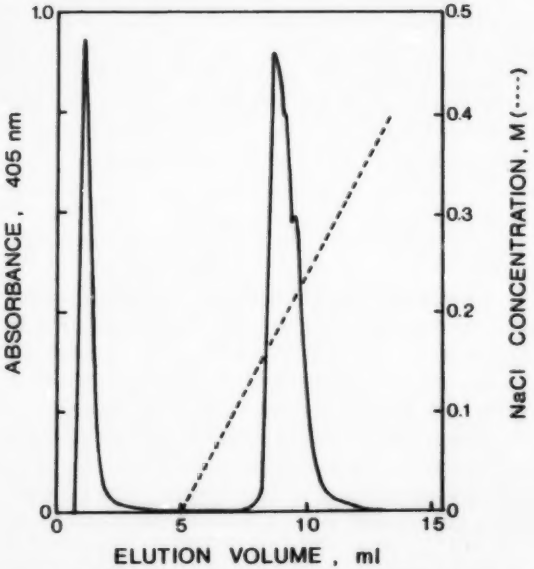

Fig. 1. Chromatogram of a 50-mg sample of G-SFH reaction mixture eluted from a HR 10/10 Polyanion SI-17 μm column (Pharmacia). Buffers A and B are described in the text. The flow-rate was 1 ml/min.

Fig. 2 shows the oxygen dissociation curve of the G-SFH preparation and ion-exchange fractions. The P_{50} of SFH is 13.5 mmHg. The oxygen dissociation curve of fraction 2 was right-shifted with a P_{50} of 26 mmHg, double that of SFH. Fraction 1 showed a smaller right-shift, with a P_{50} of 17.5 mmHg, indicating incomplete glyoxylation. The oxygen dissociation curve of the glyoxylation reaction mix-

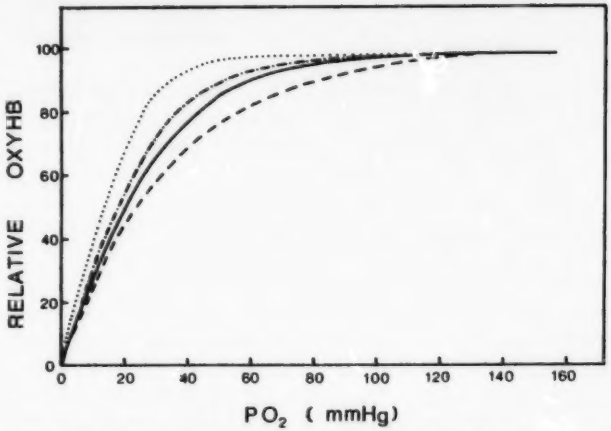

Fig. 2. Oxygen dissociation curves of SFH (·····), G-SFH reacti[illegible]ixture (———), and polyanion fractions 1 (— · — · —) and 2 (– – –) in Ringer's buffer, pH 7.4 [illegible]HB = oxygenated hemoglobin.

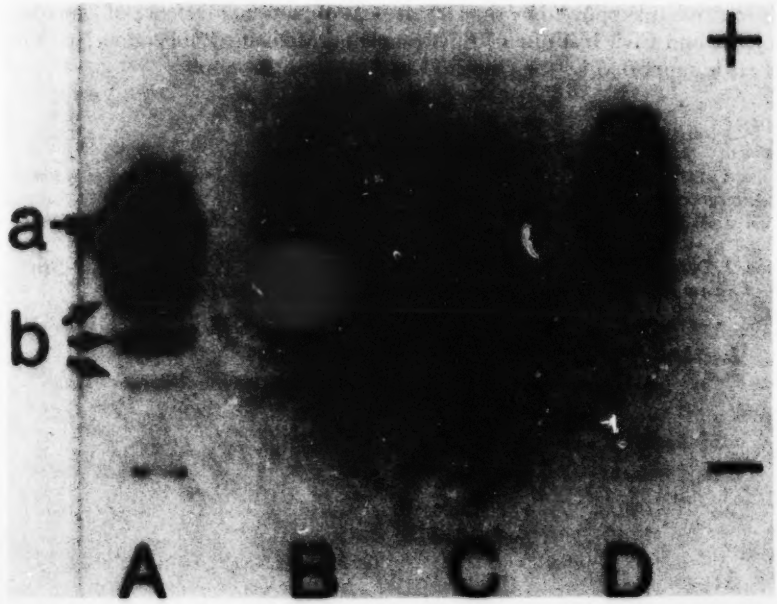

Fig. 3. Electrophoretic pattern on cellulose acetate of SFH (A), G-SFH reaction mixture (B), polyanion fraction 1 (C), and fraction 2 (D), in barbital buffer, pH 8.8. Electrophoresis was run for 45 min at 200 V on the mylar-supported cellulose acetate, which was then stained with Ponceau S. Arrow a indicates hemoglobin, arrow b indicates minor contaminants.

ture is intermediate between those of fractions 1 and 2, with a P_{50} of 20.5 mmHg, representing the average of the oxygen affinities of completely and partially glyoxylated hemoglobin.

Fig. 3 shows the electrophoretic pattern of the polyanion fractions on cellulose acetate, with SFH and G-SFH controls. Arrow a indicates hemoglobin. Fraction 2 (Fig. 3D) shows a single band with higher mobility than the major band of SFH, and does not contain the minor bands (arrows b) present in SFH, G-SFH reaction mixture, and fraction 1. This, together with the oxygen-binding data, indicates that the polyanion column separates minor impurities and partially glyoxylated hemoglobin from completely glyoxylated hemoglobin.

Thus an FPLC method achieves separation and purification of optimally glyoxylated hemoglobin from the glyoxylation reaction mixture. This technique can be used to minitor the completeness of glyoxylation in the preparation of modified hemoglobin for hemoglobin-based blood substitutes. Furthermore, these findings provide a basis for a cation-exchange method for removing partially modified hemoglobin from its reaction mixture; the minor impurities and partially modified hemoglobin will be retained by the column while the completely modified fraction passes through. This may facilitate the scaled-up production of purified glyoxylated hemoglobin.

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